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(54) Title: METAL-INTERFERON-ALPHA CRYSTALS (57) Abstract The present invention provides for crystalline zinc-interferon alpha-2 (IFN-alpha-2). The present invention further provides for crystalline cobalt-IFN-alpha-2, and crystalline IFN-alpha-2 having a serum half-life of at least about 12 hours when injected into a primate. The present invention further provides for a method for producing a crystalline IFN-alpha-2 comprising forming a soluble metal-IFN-alpha-2 complex, and equilibrating the soluble metal-IFN-alpha-2 complex in solution with an acetate salt of the metal under conditions that will cause the metal-IFN-alpha-2 solution to become supersaturated and form crystalline metal-IFN-alpha-2. The present invention also includes crystalline metal-alpha interferon having monoclinic, plate and needle morphologies.		

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METAL-INTERFERON-ALPHA CRYSTALS

5

BACKGROUND OF THE INVENTION

10 The present invention is in the field of protein crystallization and in particular protein crystallization of interferons.

The human interferon alphas are a family of proteins comprising at least 24 subspecies, Zoon K.C, Interferon 9:1 (1987), Gresser I., ed. Academic Press, New York. They were originally described as agents
15 capable of inducing an antiviral state in cells but are known as pleiotropic lymphokines affecting many functions of the immune system, Opdenakker, *et al.*, *Experimentia* 45:513 (1989). Apart from their *in vitro* biological activities the human interferon alphas are currently used for several indications, e.g., hairy cell leukemia, Kaposi's Sarcoma,
20 venereal warts, hepatitis B and hepatitis C.

Interferon alpha-2b is a purified sterile, lyophilized recombinant interferon formulation. The demand for highly purified and crystalline forms of interferon alpha, especially the recombinant type alpha-2b, is of
25 foremost importance for structure elucidation as well as for formulation of various dosage forms including the development of controlled release formulations.

Two forms of crystalline human interferon alpha have been
30 reported, namely from Miller *et al.*, *Science*, 215:689 (1982); Kung *et al.*, U.S. Patent No. 4,672,108; Weissmann, The Cloning of Interferon and other Mistakes, In: Interferon 1981, Ian Gresser, ed., Academic Press, New York, 101-134; Weissmann, *Phil. Trans. R. Soc. Lond. B*299:7 (1982); Nagabhushan, *et al.*, 'Characterization of genetically Engineered alpha-2
35 Interferon', In: *Interferon: Research Clinical Application and Regulatory Consideration*, Zoon *et al.*, Elsevier, New York 79 (1982). These publications describe methods for crystallizing interferon alpha-2 from polyethylene glycol at low temperature or from a phosphate buffer

- 2 -

solution by adjusting the pH or temperature. The Miller *et al.* article also mentions crystalline alpha-2 in a "prismatic form". Conditions for producing monoclinic prismatic crystals of interferon alpha-2b from solutions of ammonium sulfate in vapor diffusion hanging drop
5 experiments at 22°C are disclosed in International Patent Application No. PCT/US 91/03660.

IFN-alpha is generally administered either by subcutaneous or intravenous injection usually in hospital or clinical settings. IFN-alpha
10 has a serum half-life of 2-6 hours when injected subcutaneously or minutes when injected intravenously, and characteristically shows a "burst" or a "pulse" (i.e., a rapid blood serum level rise followed by a rapid blood serum level clearance) profile when blood levels are measured over time. Thus frequent administration of doses of the
15 protein must be made to maintain a therapeutically effective blood serum concentration of the drug. There are clinical situations when it may be therapeutically more advantageous to develop an IFN-alpha formulation in which the protein is continuously released into the blood stream so that the serum concentration of the protein reaches a
20 plateau and remains at that level for a period of time. This is known as a controlled release formulation.

To date none of the known crystalline IFN-alphas have shown properties desirable for a controlled drug delivery system, in particular,
25 limited solubility at 37°C and stability in a 'Generally Recognized as Safe' (GRAS) category formulation suitable for injection. There are a number of potential advantages of a controlled release therapeutic. Primarily, controlled release drugs can be administered at lower effective doses which improves their safety while maintaining or
30 improving their efficacy. New therapeutic indications can be explored because prolonged bioavailability offers the opportunity for increased biodistribution to enhance tissue and organ penetration.

There is thus a need for a controlled-release formulations of IFN-
35 alpha.

- 3 -

SUMMARY OF THE INVENTION

5 The present invention provides for crystalline zinc-interferon alpha-2 (IFN alpha-2) having a monoclinic morphology. The present invention further provides for crystalline cobalt-IFN alpha-2 and crystalline IFN alpha-2 having a serum half-life of at least about 12 hours when injected subcutaneously into a primate.

10 The present invention further provides for a method for producing a crystalline IFN alpha-2 comprising forming a soluble metal-IFN alpha-2 complex and equilibrating the soluble metal-IFN alpha-2 complex in solution with an acetate salt of the metal under conditions that will cause the metal-IFN alpha-2 solution to become supersaturated
15 and form crystalline metal-IFN alpha-2.

The present invention includes crystalline metal-alpha interferon having monoclinic, plate and needle morphologies.

20 BRIEF DESCRIPTION OF THE DRAWING

FIGURE 1 depicts serum blood level of Interferon alpha-2b in phosphate buffer solution, graph 10, and of crystalline zinc interferon alpha-2b as a function of time injected by means of a protamine sulfate
25 vehicle, graph 12.

DETAILED DESCRIPTION OF THE INVENTION

30 The present invention relates to new crystalline morphologies of metal complexes of IFN-alpha. In particular, crystalline interferon complexes with zinc and cobalt are disclosed. These crystals have desirable solubility properties for use in drug delivery systems, which include limited solubility at 37°C, particle range <200 µm and stability at room temperature in solutions suitable for injection. Using a single
35 subcutaneous injection of 34×10^6 IU of crystalline zinc-IFN-alpha-2b suspension, the measured elimination serum half-life was 12 hours as compared to 2-3 hours for the non-crystallized form INTRON A®

- 4 -

(Schering-Plough, Kenilworth, New Jersey) of IFN-alpha-2b. This is a 4-6 fold increase in serum half-life.

Supersaturated solutions of metal-interferon complexes can be induced to crystallize by several methods such as vapor diffusion, liquid diffusion, constant temperature and temperature induction or a combination thereof. Crystallization only occurs under narrow conditions of protein concentration, buffer concentration, metal ion concentration and temperature. These designated conditions for supersaturation can be obtained by vapor diffusion (hanging drop method), liquid diffusion (dialysis and ultrafiltration) at constant temperature between 4° to 22°C or via temperature induction method (temperature raised from 4° to 22°C over time). Preferably the metal salts used to complex with the interferon alpha-2b are salts of cobalt or zinc and the equilibration is carried out by constant temperature or temperature induction.

The solution of IFN-alpha-metal complex contains a metal acetate salt. The metal acetate salt is preferably selected from zinc, cadmium, potassium, lithium, magnesium and cobalt more preferably it is zinc acetate and this solution is induced to crystallize either by a constant temperature method or a temperature induction method. In the case of vapor diffusion and liquid diffusion experiments, the solution is preferably equilibrated against a more concentrated zinc or cobalt acetate solution. Equilibration refers to the process in which the solvent of one solution having a lower concentration of salt osmotically diffuses into the solution of a second solution having a higher concentration of salt in an attempt to bring the concentrations of the salt in the two solutions into equilibrium. The acetate salt is preferably present in the crystalline IFN-alpha-2 solution at the time crystals begin to form in a concentration of from about 60 mM to about 140 mM, more preferably in a concentration of from about 80 mM to about 100 mM acetate salt. As noted below, the concentration of acetate salt at the start of the equilibration procedure will be lower, i.e., from about 20 mM to about 70 mM in the case of a vapor diffusion or liquid diffusion experiment.

- 5 -

Preferably, the IFN-alpha-2 is interferon alpha-2b and is more preferably human, recombinant interferon alpha-2b. In one embodiment, the material is interferon alpha-2b having the amino acid sequence shown in Sequence ID NO: 1.

5

IFN-alpha-2a may also be employed. The primary amino acid sequence of interferon alpha-2a differs from the above sequence of IFN-alpha-2b by the replacement of lysine for arginine at residue 23.

10

The acetate salt solution of interferon alpha-2 preferably includes a buffer having a pH of 5.0 to 7.0 more preferably from 5.5 to 6.5, such as a 35 mM sodium acetate, pH 6.0 buffer solution.

15

As noted above, the method of the present invention involves preparing a metal-IFN-alpha-2 soluble complex which under designated conditions of supersaturation crystallization occurs. Conditions for supersaturation can be reached using several crystallization methods such as vapor diffusion, liquid diffusion at constant temperature and temperature induction or a combination thereof. In a vapor diffusion method, a zinc-IFN-alpha-2 complex is equilibrated against an acetate salt solution that will cause the zinc-IFN-alpha-2 solution to become supersaturated and form interferon alpha-2 crystals at constant temperature. In a liquid diffusion method, a zinc-IFN-alpha-2 complex in a zinc acetate buffered solution is dialyzed against a higher concentration of a zinc acetate buffered solution at constant temperature. In a temperature induction method, a metal-IFN-alpha solution in a metal acetate buffered solution is induced to crystallize by raising the temperature from 4°C to 22°C.

30

Any suitable IFN-alpha-2 can be employed, e.g., IFN-alpha-2a and IFN-alpha-2b, more preferably human, recombinant IFN-alpha-2a (r-h-IFN-alpha-2a) or IFN-alpha-2b (r-h-IFN-alpha-2b). Commercially available IFN-alpha-2 preparations are available from Hoffmann-La Roche (ROFERON®) and Schering-Plough (INTRON A®). Mixtures of pure interferons including IFN-alpha 2 are available from Burroughs-Wellcome Corporation (WELLFERONS®). In view of the high degree of sequence homology in the human IFN-alphas, the method of the present invention should be applicable for each subspecies.

35

The human IFN- α -2 subspecies may be obtained through recombinant DNA technology or may be purified from natural sources (e.g. human peripheral blood lymphocytes, human lymphoblastoid cell lines), for example, as described in Pestka, *et al.*, *Ann. Rev. Biochem.*, 56:727 (1987). A preferred IFN- α -2 is r-h-IFN- α -2b having the amino acid sequence of SEQ ID NO: 1.

Natural human IFN- α s have been purified from several cell sources including leukocytes isolated from whole blood, neonatal fibroblasts, lymphoblastoid and various leukemic cell lines. The first clinically available preparation of human leukocyte interferon was developed by K. Cantell and associates in Finland, in which centrifuged blood from normal donors is primed with interferon, induced to produce IFN- α by addition of Sendai virus and centrifuged. The resulting supernatant is precipitated with potassium thiocyanate, extracted with ethanol, pH precipitated, and dialyzed against phosphate-buffered saline to produce purified IFN- α , K.E. Morgensen, *et al.*, *Pharmacol. Ther.* 1:369 (1977).

Recombinant IFN- α s have been cloned and expressed in *E. coli* by several groups, for example, C. Weissmann, *et al.* *Science* 209:1343 (1980). The purification of recombinant IFN- α s has been described by several groups using a combination of chromatographic steps such as ammonium sulfate precipitation, dye affinity chromatography, ion exchange and gel filtration, for example, as described in Weissmann, C., *Phil R. Soc. (London)*, b299 :7 (1982). An alternative approach for purifying recombinant IFN- α s employs immunoaffinity chromatography with an immobilized antibody, P.P. Trotta *et al.*, *Developments in Industrial Microbiology* 72:53 (Elsevier, Amsterdam 1987). For a review of available purification schemes used for recombinant alpha interferons, see T.L. Nagabhushan and P.P. Trotta, *Ullmann's Encyclopedia of Industrial Chemistry* A14, VCH : 372 (Weinheim, Federal Republic of Germany 1989). Preferably, the IFN- α -2b used is purified by a conventional purification process described in *Ullmann's Encyclopedia of Industrial Chemistry*, followed by reverse phase high performance chromatography.

- 7 -

Suitable methods of vapor diffusion for crystallizing IFN- α include using drops, e.g., hanging or sandwiched droplets. Vapor equilibration of an acetate salt solution of metal IFN- α -2 can be effected against a second acetate salt solution that has a higher concentration of the acetate salt than the first solution. Preferably, the equilibration occurs slowly, e.g., from over 1 hour to 30 days.

Large scale crystallization may be accomplished by other methods similar to vapor diffusion to establish supersaturation, namely, liquid diffusion, e.g., dialysis and ultrafiltration. Crystallization can also be induced by temperature induction, where non-crystalline suspensions or solutions of metal-interferon become supersaturated upon raising the temperature and subsequently nucleation and crystal formation occurs. In clinical manufacturing, large scale crystallization can be used as a purification or concentration step.

The final concentration of the IFN- α -2 in the acetate salt solution at the point of crystallization, i.e., at the point of first crystal formation, can range from about 5 to about 80 mg/ml. More preferably, the concentration of IFN- α -2 is from about 5 to about 50 mg/ml. Preferably, the IFN- α -2 starting concentration is about 40 mg/ml.

In the vapor diffusion method, the concentration of the metal acetate salt in the IFN- α -2 solution at the initial stage prior to the start of crystallization can range from about 10 to about 70 mM. More preferably, the concentration of the metal acetate salt is from about 20 to about 45 mM in the interferon α -2 solution. In the counter solution at the start of the crystallization procedure, the concentration of acetate salt is from about 60 to about 140 mM, more preferably, from about 80 to about 100 mM.

The pH of the IFN- α -2 solution and the counter acetate salt solution is preferably controlled in the range of from about 4.0 to about 7.0, more preferably from about 5.5 to about 6.5. Any suitable non-metal chelating buffer can be employed for this purpose. For example, sodium acetate, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and MES (2-[N-Morpholino]ethanesulfonic acid) buffers can be employed.

- 8 -

Crystallization preferably is performed under a controlled temperature gradient for vapor diffusion and liquid diffusion methods. The temperature is preferably in the range of from about 7° to about 22°C, more preferably from about 6° to about 14°C with nucleation generally being observed at about 9°C for vapor diffusion.

For temperature induction methods, the temperature is preferably raised from 1°C to 40°C over a time period ranging from instantaneously to several days. The temperature is preferably raised from 4°C to 22°C over 1 to 10 days in a linear gradient. More preferably from 4°C to 18°C over 1 to 10 days.

The crystalline IFN- α -2 prepared by the methods of the invention will form the basis for various pharmaceutical formulations. For example, the crystalline IFN- α can be employed in a controlled release formulation, e.g. a depot preparation for subcutaneous, intramuscular, or intralesional injections capable of releasing the equivalent of a daily dose of 0.1 - 1.0 μ g/kg body weight. A depot preparation employing crystals prepared by the methods of the invention should exhibit considerably slower rate of dissolution than a formulation containing the prior art crystals produced at the lower temperature of 4°C. In particular, ambient temperature (22°C) crystals of the present invention are less temperature sensitive than crystals that require a lower temperature of formation. Preparations can contain a physiologically effective amount of the crystalline interferon α -2 in association with a conventional pharmaceutically acceptable carrier. One can envision using the controlled release effects of crystalline proteins in combination with other controlled release technologies such as microencapsulation. For example, crystalline proteins can be entrapped in Poly[dl-lactic-coglycolic] acid or liposomes.

EXAMPLES

The following examples are included to illustrate but not to limit the present invention.

- 9 -

The IFN- α -2 employed in the following examples was recombinant human interferon α -2b expressed in *E. coli* as described in Weissmann, *et al. Science*, 209:1342(1980). The cells were cultured, harvested and extracted as previously reported in Leibowitz, P. *et al.*, US Patent 4,315,852. The resulting extract was purified by a combination of conventional purification steps: ethanol extraction, matrix gel blue ligand affinity chromatography, ion exchange and gel filtration chromatography. The resulting purified IFN- α -2b preparation was dialyzed against either USP grade water or 0.1% trifluoroacetic acid solution and lyophilized as either the free base or trifluoroacetate salt respectively.

EXAMPLE 1

Production of Crystalline Zinc IFN- α -2b having a Monoclinic Morphology

Using an automated crystallization system as disclosed in Kenyon *et al.*, U.S. Patent Application No. 07/822,504 filed January 17, 1992, International Patent Application No. PCT/US92/08296 filed October 6, 1992, 6 μ l droplets containing 20 mg/ml of IFN- α -2b in 17 mM sodium acetate, 17 mM zinc acetate, pH 5.5 were hung from the upper cover of a siliconized crystallization chamber. The upper plate was placed on the greased lower assembly of the crystallization chamber over a well containing 1 ml of 35 mM sodium acetate, 35 mM zinc acetate, pH 5.5. Large monoclinic crystals were evident from 5-6 days after incubation at 22°C.

EXAMPLE 2

Production of Crystalline Zinc IFN- α -2b having a Monoclinic Morphology

In an alternative procedure, crystalline zinc IFN- α -2b having monoclinic morphology was produced. In this procedure, the zinc IFN- α -2b crystallization condition consisted of a 10 μ l droplet containing 20 mg/ml IFN- α -2b in 2.5 mM sodium acetate, 37.5 mM zinc acetate, pH 6.1 hung from an 18 mm circular siliconized cover slide. The

- 10 -

crystallization chamber, containing 1 ml of 5 mM sodium acetate, 75 mM zinc acetate, pH 6.1, was sealed to the siliconized cover slide by a bead of high vacuum grease around the rim of the crystallization chamber, thus suspending the hanging droplet above the crystallization chamber and above the acetate salt solution. Large monoclinic crystals were produced within 5-6 days after incubation at 12°C.

EXAMPLE 3

Production of Crystalline Zinc IFN-alpha-2b having a Monoclinic Morphology

In an alternative procedure, crystalline zinc IFN-alpha-2b having monoclinic morphology was produced. In this procedure, a 10 µl drop containing 20 mg/ml IFN-alpha-2b in 45 mM zinc acetate, pH 6.1 was suspended from the siliconized cover slide. The crystallization chamber contained 1 ml of 90 mM zinc acetate, pH 6.1 and was sealed with high vacuum grease to the cover slide suspending the hanging droplet above the crystallization chamber and above the zinc acetate solution. Large monoclinic crystals were produced within 5-6 days after incubation at 12°C.

EXAMPLE 4

X-Ray Diffraction Data of Monoclinic IFN-alpha 2b

For X-ray studies, IFN-alpha-2b monoclinic crystals produced according to the process of Example 1 were mounted in glass capillaries at 22°C using CuK α radiation from a Rigaku RU-300 rotating anode generator operating at 40 kV and 100 mA. The native data set was collected on a Nicolet X-100A area detector using the same radiation source.

The crystals were stable to X-ray diffraction analysis and diffracted to about 2.7×10^{-10} m (Å) resolution, but the data became much weaker at about 3.2×10^{-10} m (Å) resolution. Different batches of crystals were subjected to X-ray analysis and gave consistent results with respect to

- 11 -

morphology. The crystals index in space group $P2_1$ with cell parameters $a = 63.1 \times 10^{-10} \text{m} (\text{\AA})$, $b = 76.6 \times 10^{-10} \text{m} (\text{\AA})$, $c = 151.4 \times 10^{-10} \text{m} (\text{\AA})$, $\alpha = 90^\circ$, $\beta = 91.2^\circ$ and $\gamma = 90^\circ$. This is the first report of a metal alpha interferon having a monoclinic morphology.

5

EXAMPLE 5

Liquid diffusion crystallization method (plates)

10

In order for a crystalline suspension to have utility in a controlled release application, it must be possible to manufacture crystals in the milligram to gram scale. The current vapor diffusion in hanging drop method is not applicable to crystallize proteins at this scale. Experiments were set up to crystallize IFN-alpha-2 using a bulk dialysis method which mimicked the vapor diffusion in hanging drop method. A 0.5 ml solution of IFN-alpha-2b (40 mg/ml), 35 mM sodium acetate, pH 5.5 was dialyzed using a microdialysis bag having a molecular weight cutoff of 5000 kD (Pope Scientific Inc., Menomonee Falls, Wisconsin) against 2.7 liters of 35 mM sodium acetate, pH 5.5 at 22°C. A zinc acetate solution (0.3M) buffered to pH 5.5 was added dropwise over a two day period at 22°C. The purpose of dropwise addition was to slowly raise the zinc acetate level to 35 mM in the IFN-alpha-2b solution. A precipitate in suspension was observed after 1-2 hours of zinc acetate solution addition. The suspension was monitored microscopically daily. After 2 weeks, a few plates were observed in the suspension. The number of plates in the suspension increased daily (average size; 70 μm) until the suspension contained about 90% crystals after 3 weeks.

30

EXAMPLE 6

Liquid-diffusion crystallization method (plates)

A 0.5 ml IFN-alpha-2b solution having a concentration of 40 mg /ml of IFN-alpha-2b in 35 mM sodium acetate, pH 5.5 was dialyzed using a microdialysis bag having a molecular weight cutoff of 5000 kD (Pope Scientific Inc., Menomonee Falls, Wisconsin) against 2.7 liters of a buffer solution comprised of 35 mM sodium acetate and 35

35

- 12 -

mM zinc acetate, pH 5.5. The resulting suspension was incubated at 22°C for 3 weeks. Masses of plate crystals were evident from 3-4 weeks by microscopic inspection.

5

EXAMPLE 7Temperature induction crystallization method (plates)

10 A 0.5 ml IFN- α -2b solution having a concentration of 40 mg/ml of IFN- α -2b in 35 mM sodium acetate, pH 5.0 was adjusted to pH 6.0 using 1 M sodium hydroxide at 4°C. The resulting suspension was submerged in a refrigerated bath/circulator (model #RTE-110, Neslab Instruments, Inc., Newington, N.H.). The temperature of the water bath was increased to 22°C using a linear
15 gradient over 4 days. Masses of plate crystals were evident after 4 days by microscopic inspection.

EXAMPLE 8

20 Production of Crystalline Zinc IFN- α -2b Using a Combination of Vapor Diffusion and Temperature Induction Methods

Using a combination of vapor diffusion and temperature induction, crystalline zinc IFN- α -2b having monoclinic morphology
25 was produced. In this procedure, a 10 μ l droplet containing 20 mg/ml IFN- α -2b in 40 mM zinc acetate, pH 6.0 was suspended from a siliconized cover slide at 4°C. The crystallization chamber contained 1 ml of 80 mM zinc acetate, pH 6.0 and was sealed with high vacuum grease to the coverslide suspending the hanging droplet above the
30 crystallization chamber. The entire chamber was transferred to an incubator in which the temperature was 12°C. Large monoclinic crystals were produced within 3-5 days after incubation at 12°C.

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- 13 -

EXAMPLE 9-14

5 Characterization

Studies were initiated to characterize the zinc IFN- α -2b crystals using physical biochemical methods to insure molecular integrity, protein zinc content and retention of biological activity after
10 dissolution.

EXAMPLE 9

15 Protein Assay

An aliquot of bulk zinc IFN- α -2b crystals produced by the procedure of Example 3 was dialyzed against 2 liters of 35 mM sodium acetate, pH 5.5 at 22°C for 4 days to remove non-complexed zinc acetate. The suspension was centrifuged and the wash solution was removed
20 with a Pasteur pipette. The washed crystals were redissolved in 8 M guanidine hydrochloride solution at 22°C. Protein concentration was determined by a modified Bradford assay using pure human IFN- α -2b as a reference standard. Bradford assay: A modification of the standard Coomassie blue dye binding assay so that the absorbance is
25 directly proportional to protein concentration. Details are in Bradford, M., *Anal. Biochem.* 72:248 (1976).

EXAMPLE 10

30 HPLC

Analytical high performance liquid chromatography (HPLC) (Waters Ass., Milford, MA) was performed on an aliquot of redissolved IFN- α -2b crystals produced according to the procedure of Example 3.
35 The sample was applied to a RAININ DYNAMAX® C₄ 300 x 10⁻¹⁰m (Å) column (4.6 x 250 mm) which was subsequently eluted with a linear gradient of acetonitrile 27-72% in 0.1% trifluoroacetic acid over a 30 minute period. A Gilson variable wavelength detector set at 280 nm

- 14 -

with a sensitivity of 0.02 absorbance units was used to monitor the eluate. The retention times and chromatographic profiles of both the redissolved crystal solution and the original IFN- α -2b preparation prior to crystallization were indistinguishable.

5

EXAMPLE 11

SDS-PAGE ANALYSIS

10 Crystals harvested from a vapor diffusion in hanging drop experiment according to the procedure of Example 1 were centrifuged and washed several times to remove any soluble IFN- α . The centrifuged pellet was dissolved in a buffer containing sodium dodecyl sulfate. The resulting solution was run on a 12% sodium dodecyl
15 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Laemmli, U.K. *Nature*, 227:680 (1970) vs. a sample of IFN- α -2b. There was no apparent change in the molecular weight of the dissolved crystals vs. the control IFN- α -2b samples. Based on these results, there was no evidence of chemical or enzymatic modification of the IFN- α -2b
20 during the crystallization process or subsequent dissolution.

From the results of Examples 10 and 11 above, it can be concluded that no chemical changes or any denaturing of the protein took place during the crystallization or reconstitution.

25

EXAMPLE 12

Physical Properties of Zinc IFN- α 2b

30 The properties of the crystals produced according to the procedure of Example 1 were probed for suitability in controlled release formulations by observing microscopically their stability at 37°C (body temperature) and 4°C. Also, crystal stability was observed in a non-zinc buffer at different pH's over a period of 18 hours. The crystals were
35 found to be stable for 24 hours at 37°C and 4°C and stable between pH 5.0-6.0. This differs from the characteristics of the previous crystalline IFN- α -2b preparations, especially the crystals from Nagabhushan, *et al.*, 'Characterization of genetically Engineered α -2 interferon', In:

- 15 -

Interferon: Research, Clinical Application and Regulatory, which dissolve readily above and below pH 6.0 as well as at 4°C at pH 6.0.

EXAMPLE 13

5

Molar Ratio of Complexed Zinc vs. Interferon Content

An experiment was designed to determine the molar ratio of complexed zinc vs. IFN- α -2b. An aliquot of bulk zinc-IFN- α -2b
10 crystals produced according to the procedure of Example 3 was dialyzed against 2 liters of 35 mM sodium acetate, pH 5.5 for 4 days to remove non-complexed zinc acetate. An 8.0 M guanidine hydrochloride solution was added to the washed suspension to dissolve the complex. The resulting solution was assayed using a Bradford assay for protein
15 content. A sample of the same suspension was submitted for a zinc assay based on atomic absorption analysis. A 3.1 to 1 molar ratio of zinc ions to IFN- α -2b was found. Analysis of subsequent batches of zinc-IFN- α -2b gave a ratio of from 2 to 4 moles of zinc ions per mole of IFN- α -2b.

20

EXAMPLE 14

Cytopathic Effect Inhibition Assay

25 To determine if the crystalline IFN- α -2b retained its biological activity a cytopathic effect inhibition assay was carried out. The virus which was used was the Encephalomyocarditis virus (EMC), ATCC strain VR-129B, and was grown in monolayer cultures of Vero cells and stored frozen in Medium A. (Medium A is comprised of 950 ml of
30 Minimum Essential Medium Eagle with Earle's balanced salt solution (Gibco Inc.), 100 ml fetal bovine serum, 36 ml of 7.5% sodium bicarbonate, 20 ml of 1M HEPES Buffer in saline, 20ml of 200 mM L-Glutamine, and 10 ml of penicillin and streptomycin (10,000 unit of K-Penicillin/ml. and 10,000 μ g streptomycin sulfate/ml). Confluent
35 monolayers of FS-71 cells in tissue culture roller bottles were rinsed with Hank's balance salt solution and incubated at 37°C for 10 minutes with a 2.5% trypsin solution. The trypsin solution containing the cells

- 16 -

was diluted in Medium A such that the concentration of cells was 3.5×10^5 and used in the assay as described below.

Interferon Assay: The entire procedure for the anti-viral Bioassay
5 was done in a 96 well microtiter plate. The samples to be tested were placed into the appropriate wells and serially diluted 1:2 across the plate. On each plate, 24 wells were filled with Medium A to serve as virus and cell controls. Additionally, a laboratory standard of interferon alpha-2b containing 600 IU/ml of Interferon alpha-2b was diluted to 1 IU /ml, the
10 concentration level necessary to give a 50% protection level from viral cytopathology, was included in all assays so that the relative anti-viral activity of samples could be determined and compared across assays. Each well was then seeded with approximately 3.5×10^4 cells in 0.1 ml of Medium A. The plate was covered and incubated at 37°C, 5% CO₂ for 4
15 hours. All wells, except the cell control wells, received EMC virus at a concentration appropriate to induce 90-100% cytopathology in 16-18 hours post-infection which was approximately 1.54×10^4 plaque forming units. The plates were recovered and incubated at 37°C, 5% CO₂ until the virus control wells displayed a cytopathic effect (CPE) of at least 90%.
20 The media from each well was aspirated and the cell monolayer was strained with 0.1 ml crystal violet preparation for about 30 minutes. After the crystal violet was decanted, the plates were gently rinsed with water and allowed to air dry. The virus and cell control wells were scored from 1 to 4+ (1 = <10% CPE and 4+ = >90% CPE) by visual
25 inspection of the monolayer with and without a microscope. Samples on test plates that showed appropriate control responses were then graded. The grading of each sample well consisted of visual examination and comparison by the standard wells. The 50% endpoint for samples are determined by direct comparison to the 50% endpoint
30 for the standard by selection of the sample well(s) which match most closely. The shift in a sample's 50% endpoint as compared to that of the standard gives estimates of titer values relative to the standard. Therefore, a shift of X wells [X = (50% well No. for sample) - (50% well No. for standard)] translates to a potency of 2^X times the potency of the
35 standard.

A detailed description of the assay is provided in S. Rubinstein, P.C. Familetti and S. Petska, *J. Virol.* 37:755 (1981).

- 17 -

EXAMPLE 15**Controlled Release Potential of Zinc-Interferon alpha 2b in a Protamine Vehicle.**

5

An *in vivo* experiment was devised to test the controlled release potential of the crystalline suspension in a GRAS formulation suitable for subcutaneous injection. Using IFN-alpha-2b produced according to the procedure of Example 7, a sterile zinc-IFN-alpha-2b crystalline suspension (34×10^6 IU/dose) was prepared in 10 mM sodium acetate, 10 mM zinc acetate, 0.4 mM protamine sulfate, pH 5.5 buffer. This suspension was injected subcutaneously into the small of the back of two Cynomolgus monkeys. The interferon blood serum level was monitored as a function of time at 1, 3, 6, 10, 24, 48 and 72 hours using the cytopathic effect inhibition assay (CPE).

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15

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See graph 12 of Figure 1 which shows the IFN-alpha mean serum level of the two monkeys determined by the CPE assay as a function of time.

EXAMPLE 16**Control Study**

25

The experimental results obtained in Example 15 differ from the present experiment in which non-crystalline IFN-alpha-2b was prepared in a normal saline phosphate buffer solution. A Cynomolgus monkey received a subcutaneous injection in the small of the back at a dosage of 50×10^6 IU/injection. The interferon levels in the blood serum were measured at 0, 1, 3, 6, 10, 24, 48, and 72 hours. The data are shown graphically in graph 10 of Figure 1 which shows the IFN-alpha serum level as determined by the CPE assay as a function of time.

30

From Examples 15 and 16, it can be concluded that the use of crystalline zinc IFN-alpha in a protamine vehicle results in a prolonged detectable level of IFN-alpha in the blood serum relative to the prior art IFN-alpha administration described in Example 16. Furthermore, the

35

- 18 -

- data supports the utility of zinc interferon crystalline suspension as a controlled release formulation. The crystalline complex can be manufactured in large quantities using a process based on bulk dialysis or temperature induction. This large scale process produces crystals in the 1-200 μm size which is desirable for an injectable product (can be injected with a tuberculin syringe).

TABLE 1

Pharmacokinetic Profile for Crystalline IFN Suspension vs. Non-Crystalline IFN in Monkeys

		Figure 1 <u>Graph 10</u>	Figure 1 <u>Graph 12</u>
	C _{max}	8000	1500
	T _{max}	3	3
	AUC (tf)	20225	16812
	t _f	6	24
15	C _{max}	IU/ml	Maximum plasma concentration
	T _{max}	hr.	Time of maximum plasma concentration
	AUC(tf)	IU.hr/ml	Area under the plasma concentration-time curve from the time 0 to time of final measurable sample
20	t _f	hr	Time of final measurable sample

- 19 -

TABLE 2Serum Level (CPE) vs. Time

<u>Time (hr)</u>	<u>Figure 1 Graph 10</u>	<u>Figure 1 Graph 12 (mean)</u>
0	0	0
1	0	676
3	8000	1500
6	150	900
10	0	114
24	0	0
48	0	0
72	0	0

5

EXAMPLE 17

10

Cobalt-Interferon alpha-2b Complex Crystals

Using an automated crystallization system as disclosed in Kenyon *et al.*, U.S. Patent Application No. 07/822,504 filed January 17, 1992, International Patent Application No. PCT/US92/08296 filed October 6, 1992, a 6 μ l droplet containing 20 mg/ml of alpha-2b interferon in 17 mM sodium acetate, 22 mM cobalt acetate, pH 4.6 was hung from the upper cover of a siliconized crystallization chamber. The upper plate was placed on the greased lower assembly of the crystallization chamber over a well containing 1 ml of 35 mM sodium acetate, 45 mM cobalt acetate, pH 4.6. Crystals were evident from 5-6 days after incubation at 22°C upon microscopic inspection.

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- 20 -

EXAMPLE 18

Production of Crystalline Zinc IFN-alpha-2b using Lithium Acetate in the Crystallization Buffer

5

A 10 µl droplet containing 20 mg/ml IFN-alpha-2b in 37.5 mM zinc acetate, pH 6.1, 2.5 mM lithium acetate was suspended from the underside of a siliconized cover slide. The crystallization chamber contained 1 ml of 75 mM zinc acetate, pH 6.1, 5.0 mM lithium acetate and was sealed to the coverslide with high vacuum grease. Monoclinic crystals appeared in 5-6 days after incubation at 12°C.

EXAMPLE 19

15 Production of Crystalline Zinc IFN-alpha-2b using Potassium Acetate in the Crystallization Buffer

20 A 10 µl droplet containing 20 mg/ml IFN-alpha-2b in 37.5 mM zinc acetate, pH 6.1, 2.5 mM potassium acetate was suspended from the underside of a siliconized cover slide. The crystallization chamber contained 1 ml of 75 mM zinc acetate, pH 6.1, 5.0 mM potassium acetate and was sealed to the coverslide with high vacuum grease. Large monoclinic crystals appeared in 5-6 days after incubation at 12°C.

25 While the present invention has been described in conjunction with the specific embodiments set forth above, many alternatives, modifications and variations thereof will be apparent to those of ordinary skill in the art. All such alternatives, modifications and variations are intended to fall within the spirit and scope of the present invention.

30

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Schering Corp.

(ii) TITLE OF INVENTION: Metal-Interferon-Alpha Crystals

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Schering-Plough Corporation

(B) STREET: One Giralda Farms

(C) CITY: Madison

(D) STATE: New Jersey

(E) COUNTRY: USA

(F) ZIP: 07940

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Macintosh 6.0.8

(D) SOFTWARE: Microsoft Word 5.1a

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To be assigned

(B) FILING DATE: Herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) U.S. APPLICATION NUMBER: 08/08/024,330

(B) FILING DATE: 25-FEB-1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lunn, Paul G.

(B) REGISTRATION NUMBER: 32,743

(C) REFERENCE/DOCKET NUMBER: JB0287K

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201 822 7255

(B) TELEFAX: 201 822 7039

(C) TELEX: 219165

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 165 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Cys Asp Leu Pro Gln Thr His Ser Leu Gly Ser Arg Arg Thr Leu Met
5 10 15

Leu Leu Ala Gln Met Arg Arg Ile Ser Leu Phe Ser Cys Leu Lys Asp
20 25 30

Arg His Asp Phe Gly Phe Pro Gln Glu Glu Phe Gly Asn Gln Phe Gln
35 40 45

Lys Ala Glu Thr Ile Pro Val Leu His Glu Met Ile Gln Gln Ile Phe
50 55 60

Asn Leu Phe Ser Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu
65 70 75 80

Leu Asp Lys Phe Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Glu
85 90 95

Ala Cys Val Ile Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys
100 105 110

Glu Asp Ser Ile Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu
115 120 125

Tyr Leu Lys Glu Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg
130 135 140

Ala Glu Ile Met Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser
145 150 155 160

Leu Arg Ser Lys Glu
165

- 24 -

WHAT IS CLAIMED IS:

- 1 Crystalline zinc-IFN-alpha-2 having a monoclinic morphology.
- 5 2. Crystalline zinc-IFN-alpha-2 of claim 1 wherein the crystalline zinc IFN-alpha-2 diffracts x-rays to about $3.0 \times 10^{-10} \text{m}$ (\AA) upon x-ray diffraction analysis.
3. Crystalline zinc-IFN-alpha-2 of claim 2 wherein the
10 zinc-IFN-alpha-2 is zinc-IFN-alpha-2b.
4. Crystalline zinc IFN-alpha-2 of claim 3 wherein the crystalline zinc IFN-alpha-2 indexes in space $P2_1$ with cell parameters $a = 151.4 \times 10^{-10} \text{m}$ (\AA), $b = 76.6 \times 10^{-10} \text{m}$ (\AA), $c = 63.1 \times 10^{-10} \text{m}$ (\AA), $\alpha = 90^\circ$, $\beta = 91.2^\circ$ and
15 $\gamma = 90^\circ$.
5. Crystalline zinc-IFN-alpha-2 of claim 4 wherein the zinc-IFN-alpha-2 is zinc-IFN-alpha-2b.
- 20 6. Crystalline zinc-IFN-alpha-2 of claim 1 wherein the crystalline zinc-IFN-alpha-2 is stable for at least 24 hours at 37°C in a pharmaceutically acceptable buffer.
7. Crystalline zinc-IFN-alpha-2 of claim 1 wherein the crystalline
25 zinc-IFN-alpha-2 is stable for at least 24 hours at a temperature between 4°C and 37°C in a pharmaceutically acceptable buffer at a pH between 5-6.
10. Crystalline IFN-alpha-2 wherein said crystalline IFN alpha-2 is stable for at least 24 hours at 37°C in a pharmaceutically acceptable buffer
30 at a pH between 5 and 6.
11. Crystalline IFN-alpha-2 of claim 10 wherein said crystalline IFN-alpha-2 is stable for at least 24 hours at a temperature between 4°C and
35 37°C in a pharmaceutically acceptable buffer having a pH between 5 and 6.

- 25 -

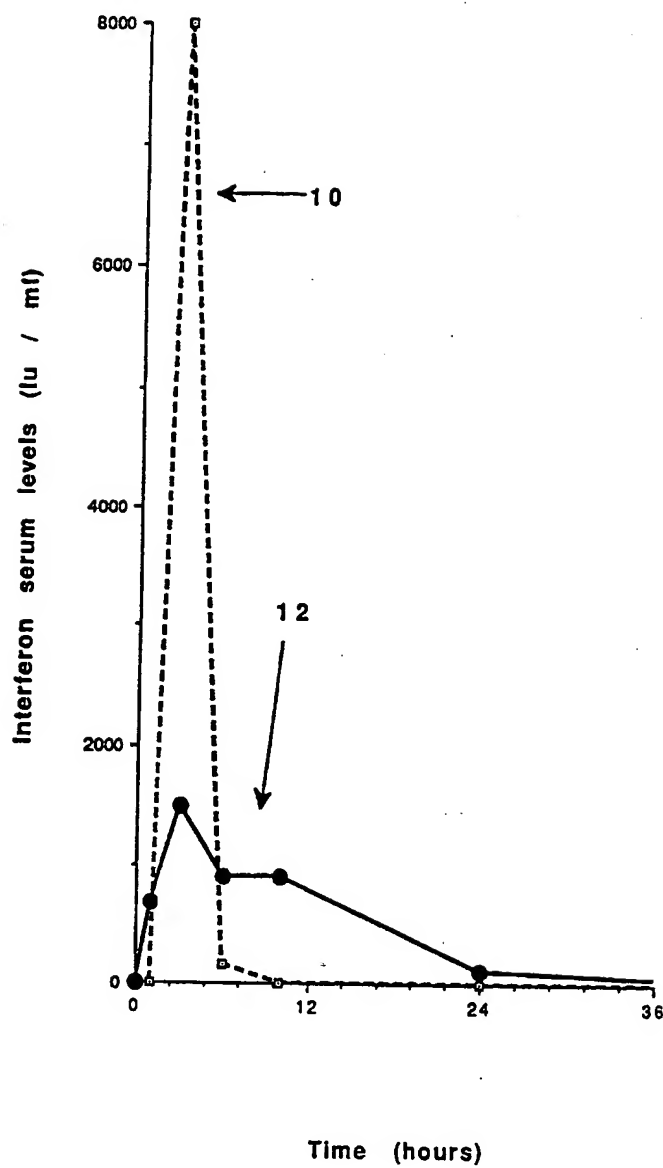
12. Crystalline zinc-IFN-alpha-2 having a molar ratio of zinc ions to IFN-alpha-2 of from 2-4 moles of zinc per mole of IFN-alpha-2.
13. Crystalline zinc-IFN-alpha-2 of claim 12 wherein the crystalline
5 zinc-IFN-alpha-2 is crystalline zinc-IFN-alpha-2b.
14. Crystalline cobalt-IFN-alpha-2.
15. Crystalline cobalt-IFN-alpha-2 of claim 14 wherein the crystalline
10 cobalt-IFN-alpha-2 is crystalline cobalt-IFN-alpha-2b.
16. Crystalline IFN-alpha-2 having a serum half-life of at least about 12 hours when injected subcutaneously into a primate.
- 15 17. Crystalline IFN-alpha-2 of claim 16 wherein the crystalline IFN-alpha-2 is crystalline zinc IFN-alpha-2.
18. Crystalline zinc-IFN-alpha-2 of claim 17 wherein the crystalline
20 zinc-IFN-alpha-2 is crystalline zinc-IFN-alpha-2b.
19. A method for administering IFN-alpha-2 to an individual comprising injecting a crystalline IFN-alpha-2 to the individual wherein the crystalline IFN alpha-2 has a serum half-life of at least about 12
25 hours when injected subcutaneously in a primate.
20. The method of claim 19 wherein the crystalline IFN-alpha-2 is crystalline zinc-IFN-alpha-2.
21. The method of claim 20 wherein the crystalline zinc-IFN-alpha-2
30 is crystalline zinc-IFN-alpha-2b.
22. A method for producing a crystalline metal-IFN-alpha-2 comprising forming a soluble metal-IFN-alpha-2 complex; and equilibrating the soluble metal-IFN-alpha-2 complex in solution with an
35 acetate salt of the metal under conditions that will cause the metal-IFN-alpha-2 solution to become supersaturated and form crystalline metal-IFN-alpha-2.

- 26 -

23. The method of claim 22 wherein the metal is selected from the group consisting of zinc and cobalt.
24. The method of claim 22 characterized by equilibration being
5 effected by means of vapor diffusion, liquid diffusion or temperature induction methods or a combination thereof.
25. The method of claim 24 wherein the metal-IFN-alpha-2 complex
10 is present in solution at a concentration of from 5 to about 80 mg/ml of solution at the point of crystallization.
26. Zinc-IFN-alpha-2 having plate morphology.
27. Zinc-IFN-alpha-2 having needle morphology.

1/1

FIGURE 1.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/01729

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K13/00 A61K37/66 C30B28/04 C30B29/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K A61K C30B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 18927 (SCHERING CORPORATION) 12 December 1991 cited in the application * page 7, line 19 - page 8, line 17; claims 14-16 *	1-27
X	EP,A,0 083 734 (F.HOFFMANN-LA ROCHE & CO.) 20 July 1983 * whole disclosure *	11,16,19
A	EP,A,0 281 299 (SCHERING CORPORATION) 7 September 1988 cited in the application	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * "&" document member of the same patent family

Date of the actual completion of the international search

7 June 1994

Date of mailing of the international search report

24.06.94

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US 94/01729

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9118927	12-12-91	AU-B- 636043	08-04-93
		AU-A- 7955291	31-12-91
		CN-A- 1058022	22-01-92
		EP-A- 0597851	25-05-94
		JP-T- 5503292	03-06-93

EP-A-0083734	20-07-83	AU-B- 551407	01-05-86
		AU-A- 9115582	16-06-83
		CA-A- 1209038	05-08-86
		JP-B- 4077000	07-12-92
		JP-A- 58164597	29-09-83
US-A- 4672108	09-06-87		

EP-A-0281299	07-09-88	US-A- 4853218	01-08-89
		AU-B- 608584	11-04-91
		AU-A- 1394688	26-09-88
		CA-A- 1316105	13-04-93
		DE-A- 3871407	02-07-92
		EP-A- 0375678	04-07-90
		JP-B- 6011712	16-02-94
		JP-T- 3500286	24-01-91
		KR-B- 9401372	21-02-94
		NO-C- 173279	24-11-93
		WO-A- 8806456	07-09-88
		ZA-A- 8801258	23-08-88

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